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Enteric Viruses Detected by Molecular Methods in Commercial Chicken and Turkey Flocks in the United States Between 2005 and 2006

Mary J. Pantin-Jackwood,[A] J. Michael Day,[A] Mark W. Jackwood,[B] and Erica Spackman[AC]

[A]Southeast Poultry Research Laboratory, USDA, Agricultural Research Service, Athens, GA 30605
[B]Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA 30677

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SUMMARY. Intestinal samples collected from 43 commercial broiler and 33 commercial turkey flocks from all regions of the United States during 2005 and 2006 were examined for the presence of astrovirus, rotavirus, reovirus, and coronavirus by reverse transcription-polymerase chain reaction (PCR), and for the presence of groups 1 and 2 adenovirus by PCR. Phylogenetic analysis was performed to further characterize the viruses and to evaluate species association and geographic patterns. Astroviruses were identified in samples from 86% of the chicken flocks and from 100% of the turkey flocks. Both chicken astrovirus and avian nephritis virus (ANV) were identified in chicken samples, and often both viruses were detected in the same flock. Turkey astrovirus type-2 and turkey astrovirus type-1 were found in 100% and 15.4% of the turkey flocks, respectively. In addition, 12.5% of turkey flocks were positive for ANV. Rotaviruses were present in 46.5% of the chicken flocks tested and in 69.7% of the turkey flocks tested. Based upon the rotavirus NSP4 gene sequence, the chicken and turkey origin rotaviruses assorted in a species-specific manner. The turkey origin rotaviruses also assorted based upon geographical location. Reoviruses were identified in 62.8% and 45.5% of chicken and turkey flocks, respectively. Based on the reovirus S4 gene segment, the chicken and turkey origin viruses assorted separately, and they were distinct from all previously reported avian reoviruses. Coronaviruses were detected in the intestinal contents of chickens, but not turkeys. Adenoviruses were not detected in any chicken or turkeys flocks. Of the 76 total chicken and turkey flocks tested, only three chicken flocks were negative for all viruses targeted by this study. Most flocks were positive for two or more of the viruses, and overall no clear pattern of virus geographic distribution was evident. This study provides updated enteric virus prevalence data for the United States using molecular methods, and it reinforces that enteric viruses are widespread in poultry throughout the United States, although the clinical importance of most of these viruses remains unclear.

RESUMEN. Virus entéricos detectados por métodos moleculares en lotes de pollos comerciales y pavos en los Estados Unidos entre 2005 y 2006.

Para determinar la presencia de astrovirus, rotavirus, reovirus, coronavirus y adenovirus (grupos I y II); se examinaron muestras de intestino tomadas de 43 lotes comerciales de pollos de engorde y 33 lotes de pavos de todas las regiones de Estados Unidos durante los años 2005 y 2006, utilizando la reacción en cadena por la polimerasa (PCR, por su sigla en inglés). Los análisis filogenéticos fueron hechos para caracterizar los virus y evaluar las asociaciones entre especies y los posibles patrones geográficos existentes. Los astrovirus fueron identificados en muestras del 86% de los lotes de pollos y del 100% de los de pavos. Tanto los astrovirus de los pollos como el virus de la nefritis aviar fueron identificados en las muestras de pollos, y a menudo los dos virus fueron detectados en el mismo lote. Los astrovirus tipo 2 y tipo 1 fueron encontrados en el 100% y el 15.4% de los lotes de pavos, respectivamente. Adicionalmente, 12.5% de los lotes de pavos fueron positivos para el virus de la nefritis aviar. Los rotavirus estuvieron presentes en 46.5% de los lotes de pollos y en el 69.7% de los de pavos evaluados. Basados en la secuencia del gen NSP4 de los rotavirus, se pudo demostrar que el origen de estos virus en pollos y en pavos es especie-específico. El origen de los rotavirus de los pavos también correspondió a la localización geográfica. Los reovirus fueron identificados en 62.8% y 45.5% de los lotes de pavos y de pollos, respectivamente. Basados en el análisis del segmento S4 de los reovirus, los virus de pollos y pavos fueron clasificados separadamente, y estos virus fueron distintos de todos los otros reovirus aviares reportados previamente. Los coronavirus fueron detectados en el contenido intestinal de los pollos, pero no en el de los pavos. Los adenovirus no fueron detectados ni en los lotes de pollos ni en los de pavos. Del total de 76 lotes de pollos y pavos evaluados, solamente tres lotes de pollos fueron negativos para todos los virus evauados en este estudio. La mayoría de los lotes fueron positivos para dos o más de los virus, y no se evidenció un patrón claro de distribución geográfica. Este estudio suministra una actualización acerca de los datos de prevalencia de los virus entéricos para Estados Unidos usando métodos moleculares, y refuerza el concepto de que los virus entéricos están distribuidos en la avicultura a través de todo el país, aunque la importancia clínica de la mayoría de estos virus aún permanece sin aclarar.

Key words: avian adenovirus, avian astrovirus, avian nephritis virus, avian rotavirus, avian reovirus, enteric virus, avian coronavirus, phylogenetic analysis

Abbreviations: aa = amino acid; ANV = avian nephritis virus; CAstV = chicken astrovirus; EM = electron microscopy; HEV = hemorrhagic enteritis virus; IBV = infectious bronchitis virus; IFA = immunofluorescence assay; nt = nucleotide; PEC = poult enteritis complex; PEMS = poult enteritis mortality syndrome; RSS = runting stunting syndrome; PCR = polymerase chain reaction; RT = reverse transcriptase; TAstV = turkey astrovirus; TCoV = turkey coronavirus

Enteric diseases cause substantial economic losses to the U.S. poultry industry because they cause decreased weight gain, increased morbidity, increased mortality, and increased production costs due to poor feed conversions. The major enteric disease complex in turkeys is poult enteritis complex (PEC) (2,14), also known as poult enteritis mortality syndrome (PEMS) in its more severe presentation (1). The last severe outbreak of PEMS with high mortality was seen in the 1990s; however, because of sporadic outbreaks of PEC, enteric disease remains a major concern for the turkey industry. Runting-stunting syndrome (RSS), also called malabsorption syndrome, is the major enteric disease complex in broiler chickens. Since 2004, the

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[C]Corresponding author. E-mail: erica.spackman@ars.usda.gov

broiler industry in the southeastern United States, and more recently in other regions of the country, has experienced severe outbreaks of RSS.

Numerous viruses, including astroviruses, reoviruses, rotaviruses, coronaviruses, and adenoviruses, have been implicated as causative agents for enteric disease because they have been isolated from or identified in the intestines and intestinal contents of affected poultry flocks (1,2,3,6,9,15,23,26,39,41). However, astroviruses and rotaviruses have also been detected in specimens from ''normal'' turkey flocks (39), making disease association difficult to determine. To our knowledge, no information has been reported on enteric viruses in normal/healthy chicken flocks.

Historically, electron microscopy (EM), immunofluorescence assays (IFAs), and genome electropherotyping have been used to detect and identify enteric viruses, or serology has been used to evaluate exposure to enteric viruses (8,13,17,28,29,30,32, 38,41,49,50). Recently, molecular methods, which have become routine in most diagnostic laboratories, have been developed for many avian enteric viruses, and they offer a more sensitive and specific alternative for virus detection and identification (6,10,16,22,46,48). Molecular methods for detecting enteric viruses offer several additional advantages over traditional methods: detection of multiple viruses in one sample, no need for virus propagation, the ability to test a large number of samples quickly, and reduced cost of the assays.

To understand and control enteric disease in poultry, more information on the prevalence and epidemiology of enteric viruses is essential and periodic surveys are necessary to develop valuable long-term data. The objective of this study was to determine, by the use of molecular tests, the prevalence of the following enteric viruses in commercial poultry in the United States: astroviruses, rotaviruses, reoviruses, coronavirus, and both group 1 and group 2 adenoviruses. To accomplish this, a survey of enteric viruses was conducted on commercial chicken and turkey operations throughout the United States. Phylogenetic analysis of the viruses detected in this study was also performed to characterize these viruses and provide epidemiological information.

MATERIALS AND METHODS

Survey design and sample collection. Commercial chicken and turkey integrators throughout the United States were contacted about voluntary participation in a survey using polymerase chain reaction (PCR)-based methods to evaluate the presence of enteric viruses in poultry regardless of the history of enteric disease within their company. All companies that were contacted agreed to participate, and instructions were provided in an attempt to standardize sample collection. The survey was conducted between September 2005 and July 2006.

Intestinal contents were collected from turkey and chicken flocks from different regions of the United States (Table 1). Samples consisted of pooled intestines from three birds per house from each of a total 43 chicken and 33 turkey farms. Sixty of the samples were from chicken or turkey flocks less than 2 wk of age; eight samples were collected from turkeys between 2 and 6 wk of age (Table 2). Information on flock condition or performance was not provided for all samples. All samples were preserved at 4 C or −70 C until shipment on wet-ice (by overnight courier) to Southeast Poultry Research Laboratory (SEPRL, USDA, Athens, GA), where samples were processed within 24 hr of receipt.

RNA extraction. Two hundred microliters of intestinal contents was diluted in 1.2 ml of phosphate-buffered saline, homogenized with sterile glass beads in a Fast-prep homogenizer (Thermo Electron Corporation, Waltham, MA), and centrifuged for 10 min at 800 × *g*. Total RNA was extracted directly from 250 μl of the supernatant using TRIzol LS reagent (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. RNA extracted from the intestinal contents of specific-pathogen-free chickens and turkeys raised at SEPRL was used as negative controls for all molecular tests.

Table 1. State and species of origin of samples examined in this study. Samples consisted of pooled intestines from three birds from a house from each of the 76 farms.

State of origin	No. of flocks	
	Chicken	Turkey
North Carolina	4	4
California	7	2
Missouri	4	15
Arkansas	10	0
Georgia	14	0
Delaware	4	0
Minnesota	0	8
Wisconsin	0	4
Total	43	33

DNA extraction. DNA was purified from samples using Tris-EDTA saturated phenol:chloroform (1:1). Briefly, 250 μl of the intestinal homogenate described under the RNA extraction methods was mixed with 250 μl of phenol:chloroform (1:1), vortexed for 10 sec, and centrifuged at 14,000 × *g* for 3 min. The aqueous phase was removed to a fresh tube. An equal volume of chloroform was added, vortexed, and centrifuged at 14,000 × *g* for 3 min. The aqueous phase was removed to a fresh tube, and 2.5–3 volumes of 95% ethanol/0.12 M sodium acetate was added, mixed by inversion, incubated on ice for a minimum of 10 min, and centrifuged at 14,000 × *g* for 15 min, at 4 C. The supernatant was decanted; 80% ethanol added, corresponding to 2 volumes of the original sample; and then samples were incubated at room temperature for 10 min. Samples were then centrifuged at 14,000 × *g* for 5 min. The supernatant was decanted, and the pellet was dried in a speed-vac (Thermo Electron Corporation) for 10 min at 37 C. Purified DNA was hydrated with 100 μl of nuclease-free water and stored long term at −70 C.

Multiplex reverse-transcriptase (RT)-polymerase chain reaction test for astroviruses. During the first phase of this study, astroviruses were detected by an RT-PCR reported by Tang *et al.* (48), that is directed to the polymerase gene (Table 3). Based on sequence data obtained from this and other studies, a multiplex RT-PCR test targeting the polymerase gene (ORF-1b), which can distinguish between the four types avian astrovirus, was designed (10). Briefly, the test has two versions: one version for turkey samples, which differentiates between turkey astrovirus (TAstV)-1, TAstV-2, and avian nephritis virus (ANV); and one version for chicken samples, which differentiates between chicken astrovirus (CAstV) and ANV (Table 3). RNA from astroviruses from the SEPRL repository was used as positive controls for the RT-PCR tests for avian astroviruses. All samples that were initially run on the first RT-PCR test (48) were re-run on the new test (10) for consistency of testing.

RT-PCR for avian reoviruses. Samples from chickens and turkeys were tested using conventional RT-PCR, instead of real-time RT-PCR, so that the identity of the amplicons could be confirmed by sequencing. Several primers designed to amplify different reovirus gene segments were tested, and a test that targets the S4 gene segment was chosen because it detected the most diverse lineages of avian reoviruses (Spackman, unpublished data). The S4 test targets a 1120-nucleotide (nt)-long region of the S4 gene (Table 3). The OneStep RT-PCR kit (QIAGEN, Valencia, CA) was used with a 25-μl reaction as follows: 1 μl of kit-supplied enzyme, 320 μM each dNTP, 0.2 μM of each primer, and 5 μl of kit-supplied reaction buffer. Thermal cycling conditions were one cycle of 50 C for 30 min, 94 C for 15 min, and then 35 cycles of 94 C for 30 sec, 53 C for 1 min, and 72 C for 1 min. RNA from two reference isolates, S1133 and NC/98, was used for the positive controls.

Table 2. Enteric virus detection in commercial chicken and turkey flocks by age.

Age (wk-days)	Astrovirus	Coronavirus	Rotavirus	Reovirus
Chickens				
Hatch to 1-0	12/14[A]	12/14	8/14	10/14
1-1 to 2-0	22/24	16/24	9/24	14/24
2-1 to 3-0	1/1	1/1	1/1	1/1
Unknown	2/2	2/2	2/2	2/2
Turkeys				
Hatch to 1-0	2/2	0/2	1/2	0/2
1-1 to 2-0	21/21	0/21	18/21	8/21
2-1 to 3-0	4/4	0/4	1/4	4/4
3-1 to 4-0	3/3	0/3	2/3	2/3
4-1 to 5-0	NA[B]	NA	NA	NA
5-1 to 6-0	1/1	0/1	0/1	1/1
Unknown	2/2	0/2	2/2	0/2

[A]Number positive/total tested.
[B]NA = not applicable, no flocks in this age range were included.

RT-PCR for avian rotaviruses. A 630-nt-long region of the NSP4 gene of rotaviruses was amplified with the NSP4 F30 and NSP4 R660 (Table 3) primers that have been reported previously (10). RT-PCR was performed using the OneStep RT-PCR kit (QIAGEN). Each 25-μl reaction contained 1× QIAGEN reaction buffer, 320 μM each dNTP, 0.2 μM each primer, 1 μl of QIAGEN enzyme blend, and 2.5 μl of extracted RNA. Thermal cycling conditions were as follows: 1 cycle of 50 C for 30 min, one cycle of 94 C for 30 min, followed by 35 cycles of 94 C for 30 sec, 55 C for 30 sec, and 72 C for 1 min. RNA from avian rotaviruses from the SEPRL repository were used as controls for the RT-PCR tests for rotavirus.

Real-time RT-PCR for coronavirus. The real-time RT-PCR test for avian coronaviruses used for this survey was conducted as reported previously (46) (Table 3), with the exception that it was run as a singlet test (the TAstV-2 primers reported in the initial test were excluded). A turkey coronavirus from the SEPRL repository was used as positive control. This test detects both turkey coronavirus and infectious bronchitis virus (IBV), but it does not detect bovine coronavirus. In addition, the previously reported real-time RT-PCR test for IBV was conducted as described previously (5) (Table 3).

PCR for avian adenoviruses. PCR tests that were described previously were used for the detection of group 1 adenoviruses (fowl adenovirus) (16) and group 2 adenoviruses (hemorrhagic enteritis virus) (47) (Table 3). Reactions were run in a 25-μl volume with 50 ng of total DNA as determined by UV spectroscopy, and with the Platinum PCR SuperMix High Fidelity kit (QIAGEN). Group 1 and group 2 avian adenovirus isolates from the SEPRL repository were used as positive controls.

Analysis of RT-PCR and PCR products. PCR and RT-PCR reactions were analyzed by agarose gel electrophoresis. Amplicons of the correct size were gel-extracted with the QIAquick gel extraction kit (QIAGEN) and prepared for sequencing as described under Sequencing.

Sequencing. Positive samples were sequenced to both confirm the RT-PCR results and to perform phylogenetic analysis. Sequence analysis was carried out with conventional RT-PCR products from the initial detection assays for astrovirus, rotavirus, and reovirus. Coronavirus sequencing template was obtained using previously described methods and primers designated IBVLC5′ (5′-ACTGGCAATTTTTCAGA-3′) and IBVLC3′ (5′-ACAGATTGCTTGCAACCAC-3′) (18).

The gene fragments used for phylogenetic analysis were as follows: the astrovirus ORF-1b from 4002 to 4425 (numbering based on the TAstV-1 genome), the rotavirus NSP4 gene from 18 to 575 (numbering based on Ty-3 NSP4 gene), and the reovirus S4 gene from 1 to 1000 (numbering based on the S1133 S4 gene). For coronaviruses, a 383-base pair product between nt 703 and 1086 relative to the start codon for the S1 gene (Massachusetts 41 virus; GenBank accession no. X04722) was amplified. The coronavirus amplified product includes the hypervariable region sequences in spike gene that have previously been shown to differentiate between IBV serotypes (19).

Direct sequencing was performed in both directions with the same primers used in the RT-PCR reactions, using the BigDye terminator kit (Applied Biosystems, Foster City, CA) run on an ABI 3730 sequencing machine (Applied Biosystems).

Phylogenetic analysis. Sequence information was compiled with the Seqman program (Lasergene 7.1; DNASTAR, Madison, WI), and the nt and deduced amino acid (aa) sequences were aligned initially with the MegAlign application of the same software package using the Clustal V alignment algorithm. Phylogenetic analysis was performed with PAUP* 4.0b10 (D. Swofford, 1998, Sinauer Associates, Sunderland, MA) using the maximum parsimony tree building method by heuristic search with 500 bootstrap replicates.

GenBank accession numbers. New sequence data used for the phylogenetic analyses were submitted to GenBank with the following accession numbers: rotavirus NSP4 gene, EU400300–EU400327; and reovirus S4 gene, EU400274–EU400299. Coronavirus sequences were identical to the Massachusetts 41 spike glycoprotein gene (GenBank accession no. AY851295). Astrovirus ORF-1b sequence data has been published (GenBank accession nos. DQ324814–DQ324836) (35).

RESULTS

Geography, flock age, and flock condition. Samples from a total of 76 flocks (43 chicken and 33 turkey flocks) from eight U.S. states with substantial commercial poultry production (Table 1) were tested for avian astrovirus, coronavirus, rotavirus, reovirus, and types 1 and 2 adenoviruses. The same types of viruses were identified in flocks from all regions of the United States, and the majority of specimens from both chicken and turkey flocks were collected from flocks less than 2 wk old. Overall turkey and chicken flocks were positive for astroviruses, rotaviruses, and reoviruses at all ages examined (Table 2).

Flock condition and performance were reported for eight chicken flocks (three ''good,'' four ''bad,'' and one ''fair'') and one turkey flock (''medium performers''). All of these flocks were positive for astrovirus, and detection of none of the other viruses correlated with reported flock condition; for example, one good and one bad chicken flock were each positive for four viruses: astrovirus, coronavirus, rotavirus, and reovirus.

Avian astroviruses. Avian astroviruses were detected in 86% of the chicken flocks and 100% of the turkey flocks tested (Table 4). Both ANV and CAstV were identified in chicken flocks, and TAstV-1, TAstV-2, and ANV were each identified in turkey flocks. Infection with multiple astroviruses was not uncommon; 38.8% of the chicken flocks and 27.9% of the turkey flocks were infected with

Table 3. Target gene, primer sequences, and amplicon sizes for the RT-PCR and PCR assays used in this study.

Target virus	Target gene	Primer name	Primer sequence	Amplicon size (bp)	Reference
Avian astrovirus	ORF-1b	TAPG-L1	5′-TGG TGG TGY TTY CTC AAR A-3′[A]	601	Tang *et al.* (48)
		TAPG-R1	5′-GYC KGT CAT CMC CRT ARC A-3′		
Avian nephritis virus	ORF-1b	ANVpol1F	5′-GYT GGG CGC YTC YTT YGA YAC-3′	473	Day *et al.* (10)
		ANVpol1R	5′-CRT TTG CCC KRT ART CTT TRT-3′		
Chicken astrovirus	ORF-1b	CASpol1F	5′-GAY CAR CGA ATG CGR AGR TTG-3′	362	Day *et al.* (10)
		CASpol1R	5′-TCA GTG GAA GTG GGK ART CTA C-3′		
Turkey astrovirus type-1	ORF-1b	T2pol2F	5′-TGG ACC GAC CCR RTT TTY ACCA-3′	251	Day *et al.* (10)
		T2pol2R	5′-GGC CCG ACY TCA GGM AGT TGT-3′		
Turkey astrovirus type-2	ORF-1b	T1pol1F	5′-AGC TYA TGM GGT TCT TTC TTC TYG-3′	911	Day *et al.* (10)
		T1pol1R	5′-GAT GGT GGG TAG CCT ATT GTG TTC-3′		
Avian reovirus	S4	S4-F13	5′-GTG CGT GTT GGA GTT TCC CG-3′	1120	This study
		S4-R1133	5′-TAC GCC ATC CTA GCT GGA-3′		
Avian rotavirus	NSP4	NSP4-F30	5′-GTG CGG AAA GAT GGA GAA C-3′	630	Day *et al.* (10)
		NSP4-R660	5′-GTT GGG GTA CCA GGG ATT AA-3′		
Turkey coronavirus and IBV	M	TCV 2F	5′-AGT GGC TTG CTA AGT G-3′	N/A[B]	Spackman *et al.* (46)
		TCV 51PB	5′-(TXRed)-TAT GCA CAC CGG ATA GAC G-(BHQ-2)-3′		
		TCV 112R	5′-GCT TTG GTC ACC AGT-3′		
IBV	5′ untranslated region	IBV5′GU391	5′-GCT TTT GAG CCT AGC GTT-3′	N/A	Callison *et al.* (5)
		IBV5′G	5′-(FAM)-CAC CAC CAG AAC CTG TCA CCT C-(BHQ1)-3′		
		IBV5′GL533	5′-GCC ATG TTG TCA CTG TCT ATT-3′		
Avian adenovirus group 1	Hexon	H1	5′-TGG ACA TGG GGG CGA CCT A-3′	1219	Hess *et al.* (16)
		H2	5′-AAG GGA TTG ACG TTG TCC A-3′		
Hemorrhagic enteritis virus	Hexon	S1F	5′-CCA GCT ACA ATC GAC ATT GAC A-3′	234	Suresh *et al.* (47)
		S2R	5′-TGT ATC TCG AAC AGG AGG CAA G-3′		

[A]IUB codes are used to denote mixed bases in primer sequences.
[B]N/A = real-time RT-PCR assay. Amplicon length not applicable.

Table 4. Number of commercial chicken and turkey flocks infected with astrovirus, coronavirus, rotavirus, reovirus, and adenovirus type-1 or type-2 infections in commercial chicken or turkey flocks as determined by RT-PCR.

	Astrovirus	Coronavirus	Rotavirus	Reovirus	Adenovirus type I or II
Chickens	37/43[A] (86.0)[B]	29/43 (67.4)	20/43 (46.5)	27/43 (62.8)	0/43 (0)
Turkeys	33/33 (100)	0/33 (0)	23/33 (69.7)	15/33 (45.5)	0/33 (0)
Total	70/76 (92.1)	29/76 (38.2)	43/76 (56.6)	42/76 (55.3)	0/76 (0)

[A]Number positive/total tested.
[B]Percentage of positive flocks.

more than one type of astrovirus (Fig. 1). The astroviruses identified in chickens were ANV, with 27 of the 34 samples positive and CAstV with 21 of 34 samples positive. Fourteen of the 34 samples were positive for both (Fig. 1). The astrovirus most frequently identified in turkeys was TAstV-2, with 23 of the 32 samples positive. TAstV-1 and ANV were identified less often (nine and four of 32, respectively), and they were always found in combination with TAstV-2 (Fig. 1).

Phylogenetic analysis was performed on most of the astrovirus-positive samples and included nt and aa ORF1b sequences available in GenBank. Part of these results have been published separately (35). Briefly, the phylogenetic assortment of the nt sequences was similar to that of the aa sequences indicating coding changes. The astroviruses assorted into four groups based on the ORF1b: TAstV-1-like, TAstV-2-like, ANV-1-like, and CAstV-like (35). There was no geographic assortment.

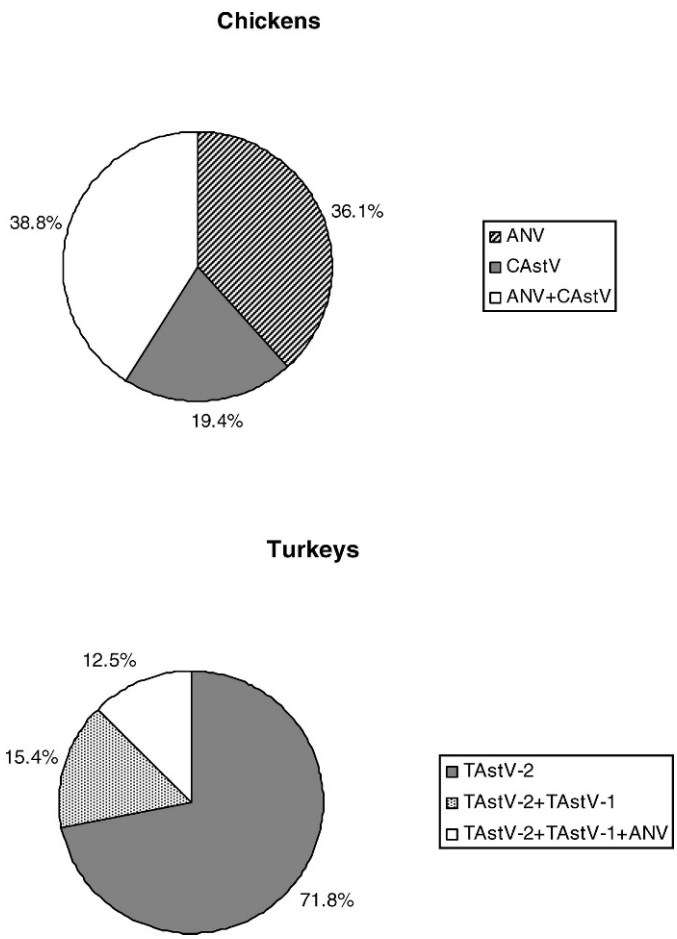

Fig. 1. Percentages of avian astrovirus types for single and concomitant infected detected in commercial chicken and turkey flocks.

Avian reoviruses. Avian reoviruses were detected in 62.8% of the chicken flocks and 45.5% of the turkey flocks tested (Table 4). There were no flocks that were infected with only reovirus (Table 5). Partial sequence of the avian reovirus S4 gene was used to determine the phylogenetic relationships among reovirus isolates collected during the survey. Sequence data of high enough quality for phylogenetic analysis was obtained from 29 of the 42 specimens. Data from the remaining 13 samples were either incomplete or not of good quality (i.e., more than 100 bp were missing); therefore, they were excluded from the final analysis.

Assortment of the reoviruses was primarily by species of origin (chicken or turkey), and there was no geographic assortment of isolates within the United States (Fig. 2). The S4 genes of turkey and chicken reoviruses had between 73.6% and 77.6% identity, whereas within genes from chicken isolates there was between 87.5% and 100% identity and between 89.9% and 100% among the turkey isolates. The viruses from this study did assort separately from all previously reported avian reovirus S4 sequence (20).

Avian rotaviruses. Avian rotavirus was identified in 46.5% and 69.7% of the chicken and turkey flocks, respectively (Table 4), and there was one flock that was infected with only rotavirus (Table 5). Phylogenetic analysis of the NSP4 gene sequence for 31 of the turkey and chicken specimens revealed species-specific and geographic assortment of the detected rotaviruses (Fig. 3). The turkey rotaviruses generally assorted based upon geographical location into groups representing North Carolina, the upper Midwest (Minnesota and Wisconsin), and Missouri. As with the majority of the chicken reoviruses, the chicken rotaviruses assorted separately from the turkey rotaviruses, and they showed little variation in NSP4 sequence identity based upon geographical location (Fig. 3). The sequence identities of the chicken-origin rotavirus NSP4 gene ranged from 89.2% to 100%. The sequence identities of the turkey-origin NSP4 gene ranged from 81.5% to 99.8%. The rotavirus sequence identity between species was as low as 62.2% and as high as 73.5%.

Coronavirus. Coronavirus was detected in the intestinal contents of 67.4% of chicken flocks and in none of the turkey flocks. BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/) of the sequence obtained from coronavirus isolate 916 showed that the isolate was identical to the Massachusetts 41 strain of IBV (GenBank accession no. AY851295).

Adenovirus. No avian adenoviruses were detected in any of the flocks tested (Table 4).

DISCUSSION

Although the types of viruses identified in this study are similar to those reported in previous surveys of turkeys and chickens (6,15,27,28,29,30,32,33,38,39,41,52), the prevalence of enteric viruses in healthy turkey flocks demonstrated in this study was much higher than reported previously. No studies on enteric viruses in healthy chickens have been reported to our knowledge. The

Table 5. Patterns of concomitant infection with astrovirus, coronavirus, rotavirus, and reovirus in commercial chicken and turkey flocks. Adenoviruses were not detected in any samples.

				No. of flocks (% of flocks)	
Astrovirus	Coronavirus	Rotavirus	Reovirus	Chicken	Turkey
+	+	+	+	12 (27.9)	0 (0)
+	+	+	−	3 (6.9)	0 (0)
+	+	−	−	4 (9.3)	0 (0)
+	−	−	−	3 (6.9)	5 (15.1)
+	−	+	−	0 (0)	13 (39.4)
+	−	−	+	3 (6.9)	5 (15.1)
+	+	−	+	8 (18.6)	0 (0)
+	−	+	+	4 (9.3)	10 (30.3)
−	+	−	−	2 (4.6)	0 (0)
−	−	+	−	1 (2.3)	0 (0)
−	−	−	−	3 (6.9)	0 (0)

higher proportion of positive flocks identified here is probably because the previous studies were conducted using EM and electropherotyping as diagnostic methods, which are not as sensitive or specific as the molecular techniques used in this study. The use of molecular tools also permitted a more in-depth characterization of the viruses by sequence analysis.

The four types of previously described avian astroviruses (TAstV-1, TAstV-2, ANV, and CAstV) (35,36) were all identified in this study. The most frequently identified astrovirus in turkeys was TAstV-2, which has a high level of genetic variation, particularly in the capsid gene (35). TAstV-1 was first isolated in 1985 (37,42) and not been reported since, was identified in nine flocks. Additionally, ANV, which is associated with a range of disease presentations from growth depression and RSS to kidney lesions (chick nephropathy) in young chickens (8,12,32,44,45), was identified in turkeys flocks for the first time. The importance of TAstV-1 and ANV infection in turkeys is still not known, and further characterization is needed.

ANV and CAstV were detected in chicken flocks, the former more frequently detected than the latter. No studies on the prevalence of these viruses in chicken flocks in the United States have been reported previously; however, CAstV has been detected by serology in broiler flocks in the United States, but no correlation between the presence of antibody and uneven growth has been demonstrated (3). ANV has been detected by RT-PCR in kidney samples from young chickens with RSS in Hungary, and phylogenetic analysis of these viruses indicated high diversity (21). The significance of detecting ANV and CAstV in normal flocks is currently unclear.

Reoviruses are known to be widely distributed in poultry (40), and most if not all flocks will probably be exposed to some avian reovirus at some point. In this report, intestinal contents were tested in an attempt to target reoviruses with an enteric tract tropism. Also, a new RT-PCR test directed to the S4 gene segment, which encodes the σNS protein (43), was used. Although more work needs to be done, based on the currently available avian reovirus sequence and these field samples, this test seems to detect more avian reovirus variants than previously reported tests directed to the S1 and S3 genes (46) and S2 gene (Spackman, unpublished data).

In this survey a substantial number of chicken and turkey flocks were positive for reovirus. The viruses assorted by species origin and none of the viruses identified here assorted with the avian reovirus reference strains such as S1133 and others that were isolated in the 1970s, 1980s, and 1990s. It is possible that this assortment pattern is due to sample bias, because there is no sequence data available for isolates between 1993 and 2005. Alternatively, the assortment may be due to some selection pressure; however, the function of the σNS protein is not known, although it is believed to have a role in virus packaging (4); therefore, it is difficult to speculate.

Rotaviruses from groups A, D, F, and G have been detected in broiler flocks based upon electropherotype analysis and serology (11,31,33) and group D avian rotaviruses, also referred to as rotavirus-like viruses, have been the most frequently reported rotaviruses in poultry (26,38,49). In a previous study using the same RT-PCR test for the NSP4 gene as used in this study, rotaviruses of four genotypes were detected in samples from commercial turkeys from hatch up to 12 wk of age (34). However, it is not known whether avian rotaviruses of the different serotypes or groups are present in these samples, because NSP4 is not a group-specific gene, and a reliable method for genetic typing of avian rotaviruses has not been established.

In the present study, phylogenetic analysis showed that the chicken-origin rotaviruses grouped with a previously reported chicken rotavirus for which NSP4 sequence data is available, Ch-1 from the United Kingdom (24). The turkey rotaviruses identified here generally grouped based upon the geographical origin of the samples, and the Minnesota and Wisconsin samples grouped with a previously described United Kingdom isolate, Ty-3 (25), and a Korean isolate, AvRv. Rotavirus was detected in chicken flocks described in the field as both good and bad performers with respect to enteric disease signs; this could be explained by the circulation of more than one avian rotavirus pathotype in poultry in the United States, in addition to the observed infections with more than one enteric virus. Additionally, age at exposure may be important for disease severity, because it has been established that in general older turkeys and chickens are more susceptible to rotavirus infection than younger birds (51). However, group D rotavirus infection has recently been implicated as a contributing factor in the development of RSS in 5- to 14-day-old broilers in Germany (33).

The coronaviruses detected in chicken samples was identical to the Massachusetts 41 strain (GenBank accession no. AY851295) and matches the aa sequence for the Mass 41 vaccine strain (Pantin-Jackwood, unpublished data). It has been reported that IBV vaccine viruses can persist in internal organs and be shed in nasal secretions and feces for up to 163 days (7). Not detecting turkey coronavirus in any turkey flocks is not unexpected, because it has been strongly associated with enteric disease, and it is relatively easy to diagnose and eradicate; so, it is rarely found in healthy turkeys.

Both types 1 and 2 adenoviruses are considered to be widely distributed in poultry (9,13,23), and not finding either was somewhat unexpected. Although it is possible that the flocks are

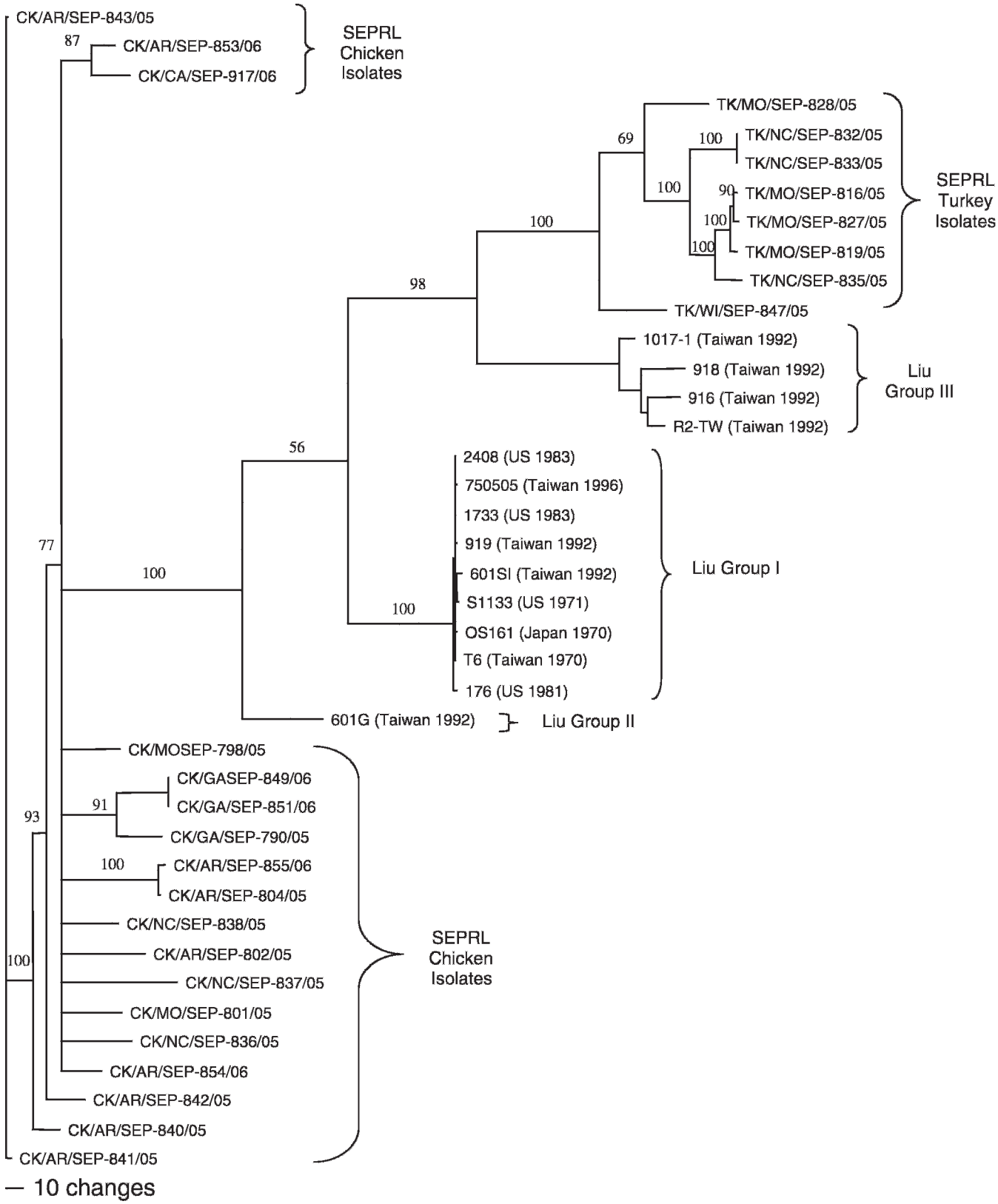

Fig. 2. Unrooted phylogenetic tree of avian reovirus S4 nt sequence from selected representative isolates and reference isolates. The tree was constructed with PAUP* 4.0b10 (Sinauer Associates, Sunderland, MA) using maximum parsimony, heuristic search, and 500 bootstrap replicates (bootstrap values are shown on tree). SEPRL isolate nomenclature is as follows: species of origin/state of origin/SEPRL accession number/year of isolation. The country of origin and year of isolation are in parentheses for reference isolates. Clades are labeled according to Liu *et al.* (20). CK = chicken; TK = turkey.

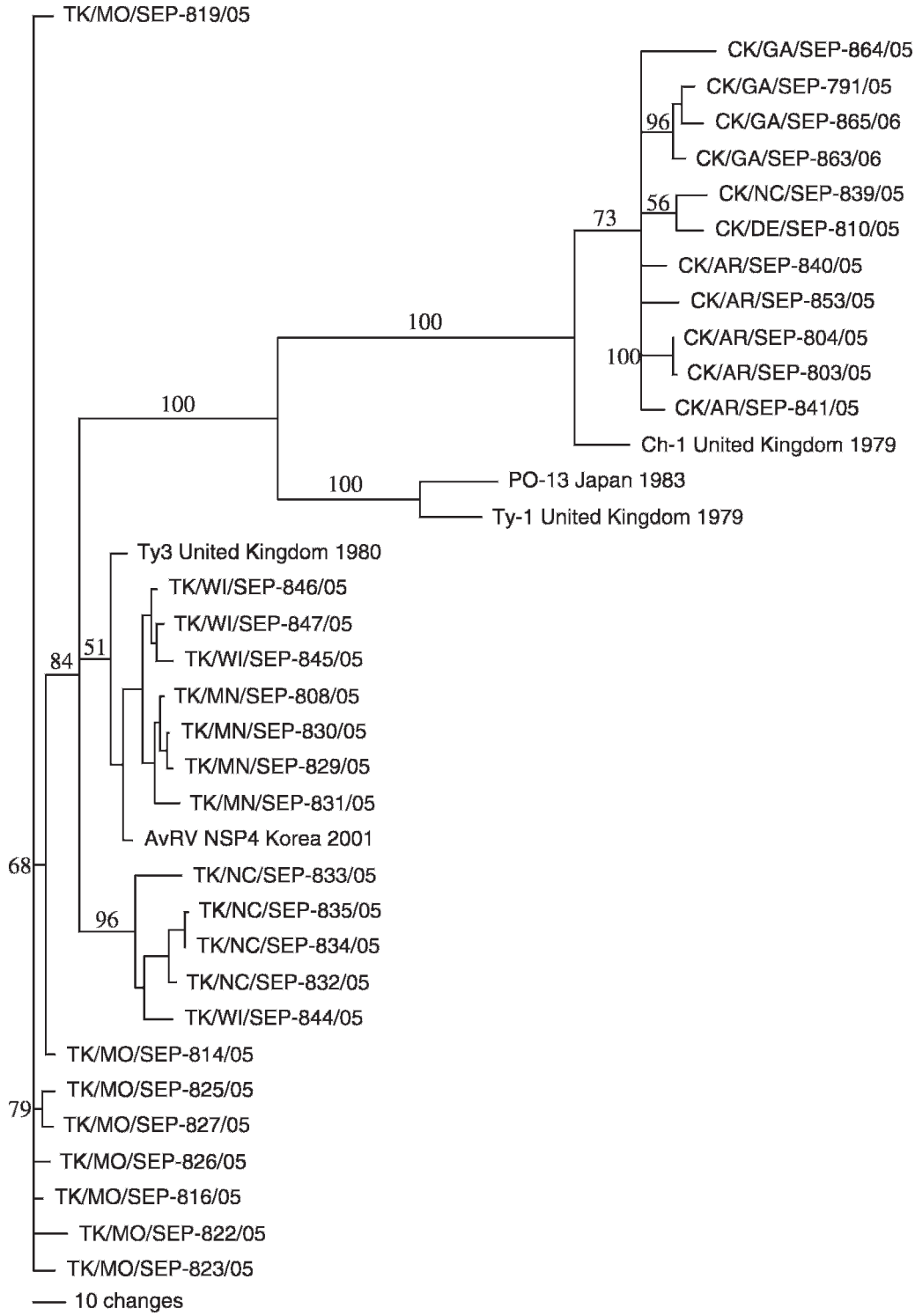

Fig. 3. Unrooted phylogenetic tree of avian rotavirus NSP4 nt sequence from selected representative isolates and reference isolates. The tree was constructed with PAUP* 4.0b10 (Sinauer Associates, Sunderland, MA) using maximum parsimony, heuristic search, and 500 bootstrap replicates (bootstrap values are shown on tree). Isolate nomenclature is as follows: state of origin/species of origin/case number/year of isolation.

all negative, another possible explanation is that the viruses in the field are variants, which are not detected by the PCR tests used.

Geographic origin, flock age, and flock condition were all considered in respect to the results. Specimens were collected from several important poultry-producing areas of the United States, and viruses of the same families were detected in flocks from all regions tested. Phylogenetically, none of the viruses assorted only by region, with the exception of rotaviruses from turkeys, indicating that the viruses are widely distributed and that regional differences in enteric disease are probably due to other factors. However, unidentified viral pathotypic variants within a family cannot be ruled out. Although astroviruses, reoviruses, and rotaviruses could be detected at all the ages tested for both turkey and chicken flocks, no conclusions can be drawn about age association, because the majority of specimens from

both chicken and turkey flocks were collected from flocks less than 2 wk old. It has been reported previously that astroviruses and rotaviruses were found to be present in turkey flocks through the life of the flock (12 wk of age; light hens) (34), indicating that enteric viruses are probably persistent in the flock or the environment through grow-out. Although conclusions cannot be drawn from reported flock condition or performance because of the subjective nature of the criteria and because of limited data, it is interesting to note that both a good flock and a bad flock could be infected with four of the same viruses. This reinforces why epidemiologic data has not been very successful in identifying viral agents that cause enteric disease in poultry.

Conclusions that may be drawn from this survey are largely in agreement with previous studies where EM or IFA were used. However, by using molecular techniques more infected flocks were identified, and the identity of the viruses could be confirmed and further characterized through sequencing. All of the viruses targeted here seem to be widely disseminated and endemic within the United States, and concomitant infection of flocks with two or more enteric viruses is common (86.0% of the chicken flocks and 84.8% of the turkey flocks).

Finally, it should be noted that as with other studies, the information presented here is only as good as the test used. Novel viruses and possibly variants of the viruses targeted here could be overlooked. Furthermore, these viruses, which have historically been identified as enteric viruses, are so widely distributed that the diagnostic value of identifying one of these viruses in a flock is limited (turkey coronavirus being the possible exception). Further work will focus on identifying specific viral factors or subtypes/subgroups associated with disease through pathogenesis studies.

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